PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3: C12N 11/10, 11/02, 11/04, 5/00, 5/02; C09H 7/00; C08B 37/08 | A1

(11) International Publication Number: WO 82/00660

(43) International Publication Date: 4 March 1982 (04.03.82)

(21) International Application Number: PCT/US81/01098

(22) International Filing Date: 14 August 1981 (14.08.81)

(31) Priority Application Number: 8005838-1

(32) Priority Date: 20 August 1980 (20.08.80)

(33) Priority Country: SE

(71) Applicant *(for all designated States except US)*: CORNING GLASS WORKS [US/US]; Corning, NY 14830 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MOSBACH, Klaus, H. [SE/SE]; Lackalaenga 31-38, S-24402 Furulund (SE). NILSSON, Kjell G.C. [SE/SE]; Fagelhundsvagen 30, S-22253 Lund (SE).

(74) Agents: MAYCOCK, William, E. et al.; Corning Glass Works, Corning, NY 14830 (US).

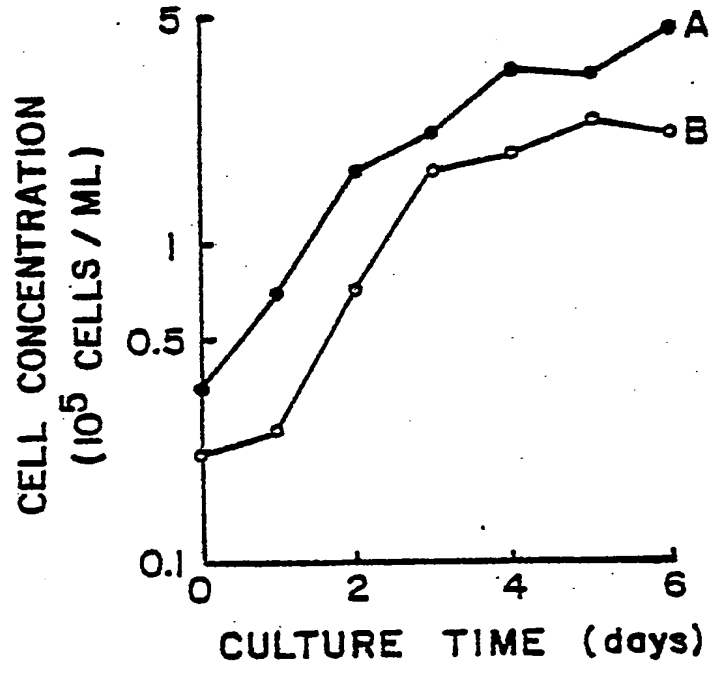
(81) Designated States: DE, FR (European patent), GB, JP, US.

Published
With international search report

(54) Title: IMMOBILIZATION OF ANIMAL CELLS

(57) Abstract

Animal cells are immobilized by adsorbing anchorage-dependent animal cells on microcarriers which can be enzymatically degraded with anzymes such as dispase and collagenase to release the cells intact. The microcarriers include naturally occurring proteins and polysaccharides or derivatives thereof, such as gelatin and chitosan. The microcarriers may be modified with a crosslinking agent such as glutaraldehyde to impart higher temperature resistance and increase mechanical strength. Incorporating particles of a magnetic material such as Fe304 in the microcarriers permits the use of an external magnetic field to stir, suspend and/or isolate the microcarriers. Alternatively, animal cells are immobilized by entrapping in alginate or agarose gels.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	KP	Democratic People's Republic of Korea
AU	Australia	LI	Liechtenstein
BR	Brazil	LU	Luxembourg
CF	Central African Republic	MC	Monaco
CG	Congo	MG	Madagascar
CH	Switzerland	MW	Malawi
CM	Cameroon	NL	Netherlands
DE	Germany, Federal Republic of	NO	Norway
DK	Denmark	RO	Romania
FI	Finland	SE	Sweden
FR	France	SN	Senegal
GA	Gabon	SU	Soviet Union
GB	United Kingdom	TD	Chad
HU	Hungary	TG	Togo
JP	Japan	US	United States of America

Description

IMMOBLILIZATION OF ANIMAL CELLS

Technical Field

The present invention relates to microcarriers. More particularly, the present invention relates to improved microcarriers and their use in the immobilization and culturing of anchorage-dependent animal cells, and to the ready removal of such cells from such microcarriers.

The surface-area limitations associated with culturing anchorage-dependent animal cells in, for example, roller bottles to a large degree have been overcome through the use of microcarriers. Where the cells per se comprise the desired product, however, problems still remain relative to the efficient harvesting of such cells.

Background Art

In the past, there has been considerable interest in the immobilization of cells, particularly those of microbial origin. K. Mosbach, Ed., "Methods in Enzymology," Vol. 44, Academic Press, New York, 1976; and I. Chibata and T. Tosa in L. B. Wingard et al., "Applied Biochemistry and Bioengineering," Vol. 1, Academic Press, New York, 1976, pp. 329-357. More recently, such interest has been extended by the reported immobilization of living plant cells in suspension. P. Brodelius et al., FEBS Letters, 103, 93-97 (1979).

The use of microcarriers for culturing mammalian anchorage-dependent cells in suspension has been given increasing attention in recent years. For example, a DEAE-Sephadex microcarrier was used in some early experiments as a support for the cultivation of fibroblast-like

cells derived from embryonic rabbit skin (H cells) and diploid human embryonic lung cells (HEL cells). A. L. van Wezel, Nature, 216, 64-65 (1967).

van Wezel has continued to study the use of DEAE-Sephadex microcarriers for the homogeneous cultivation of primary cells and diploid cell strains. See, e.g., A. L. van Wezel, "Microcarrier Cultures of Animal Cells," in P. F. Kruse, Jr. and M. K. Patterson, Jr., Editors, "Tissue Culture: Methods and Applications," Academic Press, New York, 1973, pp. 372-377; A. L. van Wezel et al., Develop. biol. Standard., 42, 65 (1979), presented at the 2nd General Meeting of ESACT, Paris 1978; and A. L. van Wezel et al., Process Biochemistry, 13, 6 (1978).

Interestingly, other workers also concentrated on the use of DEAE-Sephadex microcarriers. For example, Levine et al. proposed adding to the culture medium containing DEAE-Sephadex microcarrier beads a negatively charged non-nutritive component, e.g., carboxymethyl-cellulose, to compete with the positively charged sites on the microcarrier. They also screened various support (microcarrier) materials which appeared to include silica gel and several ion-exchange resins, as well as Sephadex per se. The DEAE-Sephadex beads were in the 90-105 μ range. D. W. Levine et al., "Optimizing Parameters for Growth of Anchorage-Dependent Mammalian Cells in Microcarrier Culture," in "Cell Culture and Its Applications," Academic Press, New York, 1977, pp. 191-216. See also U.S. Patent No. 4,036,693 to D. W. Levine et al.

Additional work by D. W. Levine et al. indicated that such microcarrier treatments as that described above can be eliminated if the charge capacity of the microcarrier is adjusted or controlled within a certain range, thereby resulting in good growth of a wide variety of anchorage-dependent cells. Such range is from about 0.1

to about 4.5 milliequivalents per gram of dry material. The microcarriers, however, still appear to be positively charged, and the most suitable microcarrier apparently is DEAE-Sephadex. U.S. Patent No. 4,189,534 to D. W. Levine et al., or British Patent Specification No. 1,535,150 to Massachusetts Instititue of Technology. See also D. W. Levine, Ph.D. Thesis, "Production of Anchorage-Dependent Mammalian Cells on Microcarriers," Massachusetts Institute of Technology, 1977.

Of course, materials other than DEAE-Sephadex have been employed as microcarriers. See, for example, U.S. Patent No. 3,717,551 to B. Bizzini et al., which discloses the cultivation of cells on porous silica spherules. Moreover, the immobilization of cells for cultivation purposes has been achieved by means other than through the use of microcarriers, such as by entrapping the cells within a polymeric or gel-like matrix. By way of illustration only, see P. Brodelius et al., FEBS Letters, 103, 93 (1979), which describes the entrapment of plant cells within alignate beads.

Finally, the use of gelatin, collagen, and related materials in the cultivation of cells is known. For example, U.S. Patent No. 4,169,761 to P. Precausta et al. discloses the cultivation of cells on collagen fibers which are dispersed or suspended in the nutrient medium. The use of collagen in the form of gel particles for the cultivation of cells on microcarrier surfaces also has been reported; the same report mentions similar uses for cellophane, cellulose sponge, and DEAE-Sephadex, among other materials. C-b. C. Horng, Ph.D. Dissertation, "Primary Culture of Mammalian Cells on Microcarrier Surface," Graduate School, State University of New York at Buffalo, 1975, pp. 4, 5, 136, and 138. See also, J. Leighton, "Collagen-Coated Cellulose Sponge," in P. E. Kruse, Jr., Ed., "Tissue Culture: Methods and Applica-

tions," Academic Press, New York, 1973, pp. 367-371 [Chem. Abstr., 81, 23770n (1974)].

According to French Patent No. 2,419,321, DEAE-Sephadex beads can be pretreated with calf fetus serum to reduce the loss of the inoculum and the lack of reproducibility in cell cultivation procedures using DEAE-Sephadex microcarriers.

It perhaps should be noted that microbial cells have been incorporated in glutaraldehyde-crosslinked gelatin to give an immobilized glucose isomerase. U.S. Patent No. 4,191,810 to N. Yoshikazu et al. Also, the fractionation and manipulation of cells have been accomplished through the use of fiber fractionation techniques - the fiber typically is nylon, optionally coated with gelatin or a gelatin derivative which permits cell recovery by the simple expedient of melting the gelatin or gelatin derivative. See, e.g., G. M. Edelman and U. Rutishauser, "Specific Fractionation and Manipulation of Cells with Chemically Derivatized Fibers and Surfaces," in W. B. Jakoby and M. Wilchek, Editors, "Methods in Enzymology," Vol. 34, Academic Press, New York 1974, pp. 195-209.

With respect to harvesting or removing cells from microcarriers, the use of trypsin is well known. See, e.g., D. W. Levine et al. in "Cell Culture and Its Applications," Academic Press, New York, 1977, p. 207; U.S. Patent Nos. 4,036,693 and 3,717,551; and French Patent No. 2,419,321.

Disclosure of Invention

It therefore is an object of the present invention to provide a microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells and having a reduced cost.

It also is an object of the present invention to

provide a microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells, from which microcarrier such cells are readily removed if required.

A further object of the present invention is to provide a magnetic microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells, which microcarrier has a reduced cost and from which microcarrier such cells are readily removed if required.

Yet another object of the present invention is to provide a method for entrapping various animal cell types in alginate or agarose gels.

Still another object of the present invention is to provide a method for the ready removal of anchorage-dependent animal cells from the microcarriers of the present invention.

These and other objects will be apparent to one having ordinary skill in the art from a consideration of the disclosure and claims which follow.

Accordingly, the present invention provides a method for the immobilization of animal cells, characterized in that anchorage-dependent cells are adsorbed on a microcarrier which is enzymatically degradable without significant destruction of cell surfaces.

The present invention also provides a microcarrier for the immobilization of animal cells, characterized in that said microcarrier is enzymatically degradable without significant destruction of cell surfaces and that upon enzymatic degradation of said microcarrier having anchorage-dependent animal cells adsorbed thereon, said cells are released intact.

The present invention further provides a method for the immobilization of animal cells capable of cell division, characterized in that said cells are entrapped in a carrier comprising alginate or agarose.

In a preferred embodiment, the microcarrier is a naturally occurring protein or polysaccharide or derivative thereof which is essentially water-insoluble at ambient temperature.

Brief Description of Drawings

Figure 1 illustrates the growth of two primary cell cultures on the gelatin beads of the present invention.

Figure 2 illustrates the release of microcarrier-bound cells using trypsin, collagenase, and dispase, respectively.

Modes for Carrying Out the Invention

In the course of our studies to obtain a suitable gel matrix for entrapment of animal cells, we found, in analogy to T. Elsdale and J. Bard, J. Cell Biol., 54, 626-637 (1972), that collagen substrate adhered to cells. On further investigation, a simple technique leading to the preparation of solid beads, 100-250 μm, of the closely related gelatin (the product obtained on boiling collagen) was developed. All the cells tested attached and proliferated. In this context, magnetic gelatin beads were also prepared and, as expected, no adverse effect on cell growth was observed. Potentially, such supports would permit facile recovery when used in media of viscous or particulate nature. Furthermore, it is conceivable to suspend the beads by applying an outer magnetic field, thereby minimizing the need for vigorous agitation when using carriers of high specific weight.

Beads of ubiquitous chitosan, a partially deacetylated product of the polysaccharide chitin, were also prepared using a similar procedure and found to lead to attachment of cells. Their properties as supports for cell growth will be tested further. In our investigations to find supports for cell growth of anchorage-dependent cells, we coupled polylysine covalently to

CNBr-activated agarose beads. Although nothing definite can be said at this point on the attachment capacity of such preparations for the cell types tested, it was found that agarose alone activated with high concentrations of CNBr did lead to attachment and growth of cells, probably due to the positive charge introduced on the matrix.

In our experiments involving detachment of cells, we attempted an alternative approach as there was the possibility with the new microcarriers used to enzymically dissolve the support directly. Both dispase and collagenase dissolved the beads, leading in the case of dispase to a clear solution. In contrast to trypsin treatment which involves destruction of the cell surface to accomplish cell removal, the approach taken here should leave the entire cells intact and viable which is an advantage, e.g., when the cells are used for immunization or when analysis of surface antigens will be done. Summarizing, as gelatin is inexpensive and represents a more 'natural' surface for cell attachment than those commercially available, it appears that such microcarriers will turn out to be valuable supports for cell growth as also strongly indicated by the spinner culture experiments reported here. The ease of cell release adds to the merits of such microcarriers. Furthermore, the microcarriers can be degraded substantially completely, thereby preventing the readsorption of the cells.

The studies reported here on the entrapment of animal cells in gels were initiated because such immobilized preparations should offer the same advantages as those observed with microorganisms or plant cells. For instance, isolated cells, when immobilized in the entrapped state, would allow convenient assay in analytical metabolic perfusion studies or could be used for the enzymic conversion of certain metabolites by analogy

to steroid transformation using entrapped bacteria [P.-O. Larsson et al., Nature, 263, 796-797 (1976)] or be utilized for, e.g., hormone production. These data demonstrate that cell entrapment leaves at least the major part, if not all, of their metabolic machinery intact. We applied particularly one of the more recently developed entrapping techniques, i.e., inclusion in calcium alginate [M. Kierstan and C. Bucke, Biotechnol. Bioeng., 19, 387-397 (1974); J. Klein and F. Wagner, DECHEMA-Monographien, 82, 142 (1979); and S. Ohlson et al., Eur. J. Appl. Microbiol. Biotechnol., 7, 103-110 (1979)].

It could be demonstrated with isolated rat hepatocytes that the cells remained at least partially intact on immobilization, as disruption of the cell membrane would have lead to leakage of the enzyme, lactate dehydrogenase. Subsequently, islets of Langerhans from mouse pancreas were immobilized by the same procedure, giving preparations still capable of insulin production/secretion. We also showed that adipocytes, entrapped in either calcium alginate or agarose, were still capable of metabolizing added radioactive glucose to fatty acids and could be stimulated further on addition of insulin.

A drawback of the calcium alginate procedure is the requirement for Ca^{2+} or similar ions to keep the network intact and, following this, the necessity to operate in phosphate-free media. Entrapment in agarose particles, as described here and applied in the studies of free fatty acid release of adipocytes, may offer an alternative approach.

With regards to anchorage-dependent cells, good microcarriers including those described here are at hand, whereas for suspension cultures entrapment appears the best alternative for immobilization. Furthermore,

anchorage-dependent cells might profit from entrapment within a three-dimensional network while adsorbed to their normal carrier as this would give some protection against, e.g., shear force. It may even be that anchorage-dependent cells for which no suitable microcarrier has yet been found may accept the polymer network as recipient attachment surface or at least remain viable as demonstrated here using trypan dye exclusion tests. Although no clear cell proliferation has yet been observed with the various entrapped cell types, they remain viable for a considerable time and permit metabolic studies, enzymic transformations or syntheses to be carried out.

The present invention is further illustrated by the example which follows. Such example, however, is not be be construed as limiting the present invention in any manner.

Example

A. MATERIALS AND METHOD

A.1. Materials

Chitosan, agarose (type VII), alginic acid (sodium salt, type IV), collagenase (type I, 190 U/mg) were obtained from Sigma. Glutaraldehyde (25%) was from Merck AG. Cytodex and Sepharose CL-6B were products of Pharmacia, Sweden, and the magnetic particles, Fe_3O_4 (5 µm), were a gift from Höganäs, Sweden. Arlacel 83 was obtained from Atlas Chemical Co., dispase (grade II, 0.5 U/mg) was from Boehringer. Gelatin (commercial grade) was from Kebo, Sweden.

A.2. Cell Types

The following cells were kindly provided by Professor O. Sjögren: the established cell lines HeLa and K562 (erythroleukemic), and the primary cell cultures S 157N (human skin fibroblasts), S 158A (human kidney carcinoma), DMH W49 (rat colon carcinoma) and DMH W1073

(rat colon carcinoma). All cells except K 562 were grown in Waymouth media supplemented with 20% fetal calf serum. K 562 were grown in RPMI medium with 10% fetal calf serum. All media were supplemented with gentamicin (50 mg/l).

A.3. Gelatin Microcarriers

A gelatin solution (10 ml; 20% (w/v)) obtained by heating to 50°C was dispersed under vigorous stirring in 100 ml of a mixture of toluene:chloroform (73/27, v/v) containing 2% (w/v) of Arlacel 83 at room temperature. After 10 min the mixture was filtered through a 100 µm nylon net whence the collected microcarriers were transferred to acetone. After careful washing with acetone the microcarriers were evaporated to dryness. In order to obtain beads which would resist higher temperatures, cross-linking with glutaraldehyde was carried out. Dry beads (1.5 g) were reswollen in 100 ml water followed by the addition of 20 ml 25% glutaraldehyde. After gentle stirring for 30 min the beads were collected and washed with 0.15 M NaCl on a 100 µm nylon net. The beads were dispersed in 0.15 M NaCl and autoclaved at 120°C for 15 min. In order to get a suitable size distribution of the beads, they were first filtered through a 250 µm nylon net and the beads in the filtrate were then collected on a 100 µm net. They were then transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min. Magnetic microcarriers were obtained by the same procedure except that in addition to 9 ml 20% (w/v) gelatin solution, 1 g Fe_3O_4 particles were added prior to dispersion in the organic phase.

A.4. Chitosan Microcarriers

A 2% (w/v) solution of chitosan in 1% formic acid was obtained by mixing chitosan with 1% formic acid and stirring overnight, followed by filtration of any undis-

solved material through a 250 µm nylon net. The chitosan solution (18 ml) was mixed with 2 ml 25% glutaraldehyde and dispersed under vigorous stirring in the same organic phase as described for gelatin microcarriers. After 15 min the mixture was transferred to methanol. The beads were then washed on a glass filter with methanol and subsequently with 0.15 M NaCl. After autoclaving at 120°C for 15 min (to obtain more rigid beads), the preparation was first filtered through a 250 µm nylon net after which the beads in the filtrate were collected on a 100 µm nylon net. The beads were transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min.

A.5. Test for Cell Growth

Before use, all microcarriers were washed with 10 vol. medium. Gelatin and chitosan microcarriers (0.25 g wet wt) were mixed with 2.5 X 10^5 cells (S 157N, S 157A, DMH W49 and DMH W1073, respectively) in 5 ml media in 10 ml siliconized test tubes. They were placed in a CO_2-incubator (5%) at 37°C and mixed in an end-over-end shaker. The cultures were checked daily and the medium was replaced if necessary. The gelatin microcarriers containing Fe_3O_4 were tested with DMH W49. Sepharose CL-6B beads activated with different amounts of CNBr were placed in 20 mm Petri dishes and mixed with 2.5 X 10^5 DMH W49 cells in 3 ml medium. The cells S 157N and DMH W1073 were allowed to grow on gelatin microcarriers (1 g beads, wet wt) suspended in 50 ml spinners. The starting cell concentrations were 35,000/ml and 22,000/ml, respectively; 60% of the medium volume was replaced each day. For determination of the cell number, duplicate samples of 1 ml suspension were taken. After washing with phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, the beads were incubated with trypsin (0.1% in PBS with 0.02% EDTA and without Ca^{2+} and Mg^{2+})

for 15 min at 37°C and the released cells counted in a Bürker chamber.

A.6. An Alternative Method for Harvesting of Cells

After 6 days the spinner culture of DMH W1073 was divided into 3 equal fractions after washing as above. They were incubated with 3 ml enzyme solution, trypsin as described above and collagenase (2 mg/ml) in PBS with Ca^{2+} but without Mg^{2+} and dispase (4 mg/ml) in Waymouth medium at room temperature. Samples were taken at intervals and the cell concentration determined.

A.7. Alginate Immobilization

Alginate (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. The cell alginate suspension was extruded through a 0.8 mm nozzle into a solution of 25 mM Hepes, 50 mM $CaCl_2$, 75 mM NaCl (pH 7.4), whereby beads with av. diam. 2 mm were formed. Immobilization was carried out at room temperature. After 5 min the beads were washed with medium and transferred to a spinner flask, which was placed in a CO_2-incubator at 37°C. At intervals beads were withdrawn and dissolved in 0.1 M EDTA (pH 7.4) and the cells were counted.

A.8. Agarose Immobilization

Agarose (1 part 4% (w/v)) dissolved in 25 mM Hepes, 125 mM NaCl (pH 7.4) placed in a waterbath at 37°C was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. This solution was made into beads by moulding it in a form made of Teflon. Solution was poured over a Teflon plate tightly covered with 3 mm holes. Another plate was used as support and the two were held together by clamps. Before moulding, the form was warmed to 37°C. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out. The

beads were put in medium in a spinner flask and placed in a CO_2-incubator at 37°C. At intervals beads were taken out and dissolved by heating to 70°C and the cells were counted.

A.9. Preparation of Entrapped β-Cells of Islets of Langerhans

Ninety isolated islets of Langerhans (av. diam. ~ 0.1 mm) were prepared from rat [I. Lundquist, Enzyme, 12, 647-657 (1971)] pancreas and washed with Krebs-Ringer buffer lacking phosphate [I. Lundquist, supra]. The cells were suspended in 0.125 ml buffer and mixed with 0.5 ml 2.5% (w/v) sodium alginate in the same buffer. The cell alginate suspension was extruded into a solution of the buffer containing 50 mM $CaCl_2$. After 15 min the beads were washed 3 X 10 ml with the buffer. Visual inspection showed that the beads contained an average of one islet/bead.

The scintillation vials were each filled with 10 beads and 2 ml buffer, gassed for 1 min with carbogen (95% O_2, 5% CO_2) and preincubated for 30 min at 37°C in a waterbath with shaking. After addition of 1 mg glucose to the vials, they were gassed and 0.1 ml samples taken after 0, 30, 60 and 120 min. The samples were frozen and stored until analyzed for insulin using radioimmunoassay. Visual inspection showed no release of the islets into the medium.

A.10. Preparation of Entrapped Adipocytes

Rat adipocytes were prepared according to J. Gilemann, Diabetologia, 3, 382-388 (1967). For entrapment in alginate the cells were transferred to 20 mM Hepes, 130 mM NaCl, 2% (w/v) albumin (pH 7.4) mixed with an equal volume of 2% (w/v) alginate (autoclaved for 15 min at 120°C) and extruded into a solution of 25 mM Hepes, 50 mM $CaCl_2$, 75 mM NaCl (pH 7.4). After 5 min the beads were washed with Krebs-Ringer buffer con-

taining 24 mM Hepes, 0.55 mM glucose and 1% (w/v) albumin (storage medium).

With entrapment in agarose the cells were transferred to the above storage medium containing 2% (w/v) albumin and mixed with an equal volume of 6% (w/v) agarose. Beads were prepared as in section 2.8. After gelling the beads were transferred to storage medium.

B. RESULTS

B.1. Cell Attachment and Growth

In a preliminary test, various cells were tested for their capacity to attach to different microcarriers (Table 1). All cells would attach to the various beads. Studies on cell growth were subsequently carried out with the most promising microcarrier, gelatin beads with the cells S 157N and DMH W1073. Upon plotting cell concentrations for the two primary cell cultures S 157N and DMH W1073 grown on gelatin beads versus culture time, it is seen that good growth occurred with no indication of cell death. Such plot is shown in Figure 1, which is based on the mean of two cell counts; curve A is for S 127N and curve B is for DMH W1073.

Table 1

Attachment of primary cell cultures to different microcarriers

	S 157N	S 158A	DMH W49	DMH W1073
Gelatin	+	+	+	+
Gelatin (magnetic)	n.t.	n.t.	+	n.t.
Chitosan	+	+	+	+
CNBr-activated Sepharose CL-6B	n.t.	n.t.	+	n.t.

n.t., not tested

B.2. Harvesting of Cells

In Figure 2, the results of three different enzymic procedures leading to cell detachment from gelatin microcarriers are given. As seen, both collagenase (curve A) and dispase (curve B) gave rise to a far higher total cell concentration than normally applied trypsin treatment.

B.3. Cell Entrapment

I. Isolated cells: In a preliminary study to test whether animal cells would survive entrapment in a gel matrix, hepatocytes from rats were isolated [C. H. Floren and A. Nilsson, Biochem. J., 168, 483-494 (1977)] and entrapped in calcium alginate. The obtained beads were subsequently incubated in perfusion buffer and water, respectively. Release of lactate dehydrogenase was monitored. About 10-times lower leakage of the enzyme was found in the sample kept in the perfusion buffer compared to the sample in water. Subsequent incubation in water of the beads previously kept in perfusion buffer, however, led to considerable leakage of active lactate dehydrogenase. Subsequently, islets of Langerhans containing β-cells were isolated and entrapped following the same procedure to check whether the latter were capable of insulin production/secretion in the immobilized state. Over 0, 30, 60 and 120 min, samples were withdrawn from the medium and assayed for insulin content using radioimmunoassay analysis. The amount of insulin secreted was 1, 23, 40 and 78 immunoreactive insulin units (μU/ml), respectively.

Finally adipocytes were isolated and entrapped in both Ca-alginate as well as agarose. They were subsequently tested for their capability to incorporate [3-^{3}H] glucose into lipids [A. J. Moody et al., Horm.

Metab. Res., 6, 12-16 (1974)] following insulin stimulation as well as for their ability to release free fatty acids by noradrenaline stimulation [N. O. Nilsson and P. Belfrage, J. Lipid Res., 20, 557-560 (1979)]. It was found that, at the outset, the immobilized adipocyte preparation showed a higher basal incorporation than free cells which was more pronounced with cells immobilized in Ca^{2+}-alginate. On stimulation with insulin, both preparations showed an increase in glucose incorporation (when higher concentrations of alginate were used, the cells did not respond to insulin in the glucose test). The free fatty acid release was only studied with agarose-immobilized adipocytes as the alginate beads were too soft. The immobilized cells also showed a response to added noradrenaline by releasing free fatty acids after about twice the time required for free cells, and at ~1/3rd the rate for free cells.

II. Cell cultures: A number of cell types, i.e., fibroblasts, HeLa, DMH W49 and K 562, the latter growing in suspension, were entrapped in gels to test whether they would grow within a gel-matrix or remain viable. The gels prepared were beads of: (a) 2% alginate; (b) 2% agarose; (c) a mixture of 2% agarose and 0.5% alginate; and (d) 2% agarose containing simultaneously entrapped Cytodex particles to which cells already had been attached. In all cases serum was entrapped simultaneously with the cells as in-diffusion of some of the components of serum was likely to be hindered. No proliferation of cells tested was observed under the conditions used; however, on average, 10-30% of the cells remained viable as judged by the trypan blue exclusion test, after 1 week of incubation. In controls run with HeLa

cells, non-entrapped but kept free in solution in siliconized tubes, they had disappeared after 1 day.

It is to be understood that the foregoing detailed description is given merely by way of illustration and that many variations may be made thereon without departing from the spirit and scope of the invention.

Claims

1. A method for the immobilization of animal cells, characterized in that anchorage-dependent cells are adsorbed on a microcarrier which is enzymatically degradable without significant destruction of cell surfaces.

2. The method of claim 1, characterized in that said microcarrier is a naturally occurring protein or polysaccharide or derivative thereof which is essentially water-insoluble at ambient temperature.

3. The method of claim 2, characterized in that said microcarrier is gelatin or chitosan.

4. The method of claim 1, 2, or 3, characterized in that said microcarrier is modified with a crosslinking agent to impart higher temperature resistance and increased mechanical strength.

5. The method of claim 4, characterized in that said crosslinking agent is glutaraldehyde.

6. The method of claim 1, 2, or 3, characterized in that said microcarrier contains particles of a magnetic material.

7. The method of claim 4, characterized in that said microcarrier contains particles of a magnetic material.

8. The method of claim 5, characterized in that said microcarrier contains particles of a magnetic material.

9. The method of claim 6, characterized in that said magnetic material consists essentially of Fe_3O_4.

10. The method of claim 7, characterized in that said magnetic material consists essentially of Fe_3O_4.

11. The method of claim 8, characterized in that said magnetic material consists essentially of Fe_3O_4.

12. A microcarrier for the immobilization of animal cells, characterized in that said microcarrier is enzymatically degradable without significant destruction of cell surfaces and that upon enzymatic degration of said microcarrier having anchorage-dependent animal cells adsorbed thereon, said cells are released intact.

13. The microcarrier of claim 12, characterized in that said microcarrier is a naturally occurring protein or polysaccharide or derivative thereof which is essentially water-insoluble at ambient temperature.

14. The microcarrier of claim 13, characterized in that said microcarrier is gelatin or chitosan.

15. The microcarrier of claim 14, characterized in that the microcarrier is modified with a crosslinking agent to impart higher temperature resistance and increased mechanical strength.

16. The microcarrier of claim 15, characterized in that said crosslinking agent is glutaraldehyde.

17. The microcarrier of claim 12, 13, 14, 15, or 16, characterized in that the microcarrier contains particles of a magnetic material.

18. The microcarrier of claim 17, characterized in that said magnetic material consists essentially of Fe_3O_4.

19. The microcarrier of claim 12, 13, 14, 15, or 16, characterized in that the microcarrier is degradable by dispase or collagenase.

20. The microcarrier of claim 17, characterized in that the microcarrier is degradable by dispase or collagenase.

21. The microcarrier of claim 18, characterized in that the microcarrier is degradable by dispase or collagenase.

22. A method for the immobilization of animal cells which are capable of cell division, characterized in that said cells are entrapped in a carrier comprising alginate or agarose.

23. The method of claim 22, characterized in that said cells are β-cells of islets of Langerhans and are utilized for the production of insulin.

K. Mosbach Et Al.
W.E.M.

SHEET 1 of 1

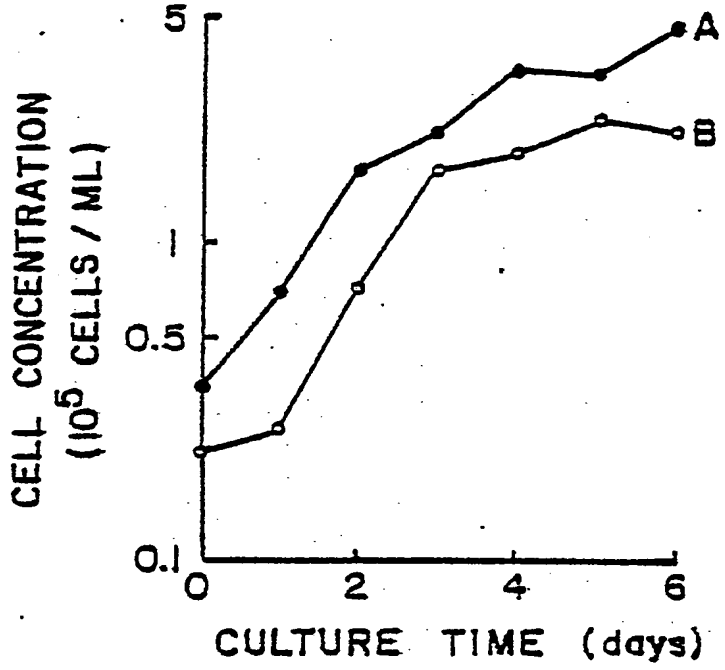


Fig. 1

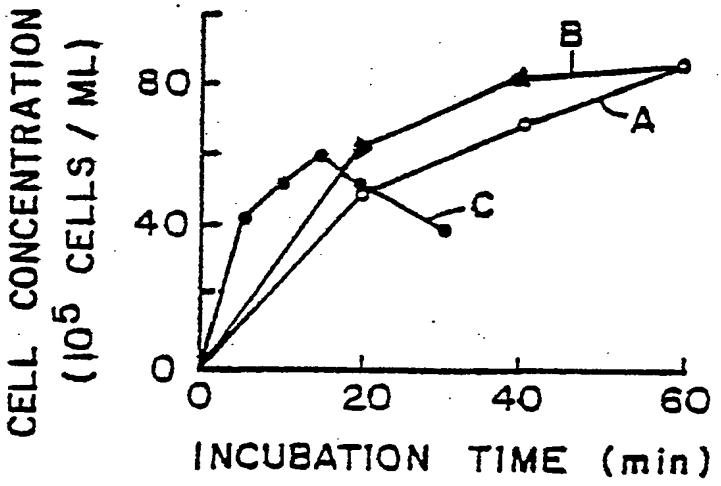


Fig. 2

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
	of Enzymes, cell contents, Cells, Va ccines, Antigans, Antiserum, Cofactors, Hormones and proteins, pages 69 and 80-87.	1-23
A	N, Chemical Abstracts, Vol. 91, Abstract no. 87596z, Issued 10 September 1979, Brodelius et al, Immobilized plant cells For The production and Transformation of Natural products.	22,23
A	N, Chemical Abstracts, vol. 81, Abstract No. 23770n, Issued 05 August 1974, Leighton, J., Collagen-coated cellulose sponge.	1-21
A	N, Chemical Abstracts, vol. 77, Abstract No. 123496p, Issued 06 November 1972, clestor, et al, probable Role of Collagen when Used As A Growth Surface For Cell culture.	1-21
A	N, Chemical Abstracts, Vol. 88, Abstract No. 132276w, Issued 08 May 1978, Dunn, et al, . Contact Guidance On Oriented Collagen Gels.	1-21
A	N, Enzyme Microb, Technol., Vol. 2, Issued January 1980, Halling, et al, Magnetic Supports For Immobilized Enzymes And Bioaffinity Adsorbents. Pages 2-11.	6-11, 17, 18
A	N, Biotechnology and Bioengineering, Vol. XIX, Issued 1977, Kierstan et al, The Immobilization of Microbial Cells, subcellular Organelles and Enzymes In Calcium Alginate Gels, pages 387-397.	22,23

INTERNATIONAL SEARCH REPORT

International Application No PCT/US81/01098

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] C12N 11/10, 02, 04; 5/00, 02; C09H 7/00; C08B 37/08
U.S. CL. 435/178, 176, 177, 182, 240, 241; 260/117; 536/20

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/174, 176, 177, 178, 180, 182, 240, 241; 260/117; 536/20

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Chemical Abstracts - Animal Tissue Culture 1972 - June 1981

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 4,169,761, Published 02 October 1979, Precaustz, et al	1-21
A	US, A, 3,972,776, Published 03 August 1976, Vieth, et al	1-21
A	US, A, 3,977,941, Published 31 August 1976, Vieth, et al	1-21
A	US, A, 3,838,007, Published 24 September 1974, Van Velzen, A.G.	1-21
A	US, A, 4,163,691, Published 07 August 1979, Devos, et al	1-21
A	US, A, 3,717,551, Published 20 February 1973; Bizzini et al	1-21
A	US, A, 4,167,447, Published 11 September 1979, Masri, et al	3-5, 14,15
A	US, A, 4,266,029, Published 05 May 1981, Branner Jorgensen, S.	1-21
A	US, A, 4,024,020, Published 17 May 1977, Weiss, et al	1-21
A	US, A, 4,266,032, Published 05 May 1981, Miller, et al	1-21

* Special categories of cited documents: [15]

"A" document defining the general state of the art
"E" earlier document but published on or after the international filing date
"L" document cited for special reason other than those referred to in the other categories
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but on or after the priority date claimed
"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention
"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
23 October 1981	27 OCT 1981
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	David M. Naff

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US, A, 4,036,693, Published 19 July 1977, Levine, et al	1-21
A	US, A, 4,189,534, Published 19 February 1980, Levine, et al	1-21
X	US, A, 3,639,558, Published 01 February 1972 Csizmas L., See col. 4, lines 31-34	12-21
X	US, A, 4,257,884, Published 24 March 1981 Lim, F., See col. 4, lines 35-57	22,23
A	N, Biomedical Applications of Immobilized Enzymes and Protein, vol 1, plenum press, Issued 1977, Chang , T.M.S. Encapsulation	
	*(cont'd on attached Form PCT/ISA/210)	

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.